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Consumption of cruciferous vegetables has been shown to be associated with decreased incidence of many cancers including the breast. Indole-3-Carbinol, one of the active compounds derived from cruciferous vegetables has been shown to arrest the growth of hormone responsive and unresponsive breast cancer cells. I3C has also been to cause increased growth arrest of MCF7 breast cancer cells in the presence of tamoxifen, an antiestrogen known to cause cell cycle arrest in MCF 7 cells. We report here that I3C causes a decrease in levels of functional ER α, without altering levels of ER β. Treatment with I3C causes a transcriptional downregulation of ER a. I3C causes an inhibition of estradiol inducible gene expression, as well as estrogen inducible total ERE activity. We also demonstrate that levels, subcellular localization, and ERE binding of ER β remain unaltered.

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Introduction

Estrogens play a critical role in development of the normal breast and in the genesis of breast cancer (1,2). Estrogens are steroid hormones, which bind and activate estrogen receptors alpha and beta (ER-α and β) in target cells. Binding of estrogens to these receptors induces dimerization. Activated receptor dimers, along with coregulators, bind specific sequences called estrogen response elements (ERE) in target gene promoter, leading to modulation of target gene expression. ER-α and ER-β are encoded by different genes, and have been shown to play different roles in the reproductive physiology of mammals (3). A high ER-α: ER-β ratio seems to correlate well with high levels of cellular proliferation, whereas predominance of functional ER-β over ER-α correlates with lower levels of proliferation (4,5). Overexpression of ER-α in estrogen sensitive breast cancer cell lines such as MCF-7 and T47D leads to no major changes in proliferation rate, whereas overexpression of ER-β in these cells leads to drastic reduction in cell growth rates in culture as well as in nude mouse xenografts (6,7). This also correlates well in the physiological setting, where during periods of intense proliferation (prepubertal phases), ER-α expression predominates, and during lactation, phase with no mammary epithelial mitogenesis, expression of ER-β is three times as much as ER α in the mammary epithelium (8).

ER-α expression is an important factor for prognosis of breast cancer as well. ER-α positive breast cancers correlate well with a differentiated phenotype and majority of them respond well to anti-hormonal therapy (9). Tamoxifen, a mixed antiestrogen, has been improving the survival of women with ER-α positive breast cancer. This antiestrogen is also clinically useful in treatment of ER-α positive metastatic breast cancer, and in prevention of breast cancer. Tamoxifen causes a transcriptionally inactive conformation of ER-α, and impairs entry of cells into S phase (9). However, long-term treatment with tamoxifen is associated with tamoxifen resistance, increased endometrial cancer risk and other undesirable side effects (9). Tamoxifen resistant breast cancer responds well to the antiestrogen ICI 182,780 (Faslodex). Faslodex has been shown to impair ER-a by causing proteasome-dependent degradation of ER-α (10). Many other antiestrogens are now in clinical trials for breast cancer treatment. There is now a strong demand for a therapeutic that is effective against several different breast cancer phenotypes with minimal side effects.

A number of epidemiological evidence exists to suggest that increased consumption of fresh vegetables and fruits can decrease risk for many cancers (11). Phytochemicals are emerging as a new class of promising cancer therapeutics, with possibly reduced side effects than present therapy options. Indole-3-Carbinol is one such phytochemical derived from cruciferous vegetables of the Brassica genus. I3C and its natural dimer, 3'-3' Dindolyl methane (DIM) have been shown to cause growth arrest of both estrogen sensitive MCF7 an estrogen insensitive MDA-MB-231 breast cancer cells by distinct mechanisms (12). I3C causes a transcriptional downregulation of cyclin dependent kinase 6 (CDK6), and a decrease in CDK2 kinase activity in both MCF 7 and MDA-MB-231 cells (13). Indole-3- Carbinol has also been shown to cooperate with tamoxifen in causing increased G1 cell cycle arrest in MCF 7 cells compared to tamoxifen alone. I3C

and tamoxifen however act by distinct mechanisms to bring about these effects (13). I3C has been shown to elicit antiestrogenic effects, and decrease phosphorylation of ER-α, which is thought to be central to estrogen independent activity of ER-α (14).
To date, the effects of I3C on ER- α and ER- β expression and activity remains unknown and forms the focus of this investigation. We report that at doses of I3C effective in causing G1 cell cycle arrest, I3C causes a drastic reduction of ER- α expression in MCF 7 breast cancer cells. This effect is at a transcriptional level and I3C causes reduction of promoter activity of ER-α. We also report that this downregulation leads to reduced expression of estrogen responsive genes such as progesterone receptor. I3C induced downregulation of ER-α is paralleled by a reduction in overall ERE activity as well as binding to ERE. In sharp contrast, expression and activity of ER-b remains unaltered upon I3C treatment. This is well in agreement with the hypothesis that increased relative levels of ER-β to ER-α in an estrogen responsive cell causes reduction of proliferation.

Materials and Methods

Materials:
DMEM, FBS, Calcium and Magnesium free PBS, L-Glutamine, and Trypsin-Versene mixtures were purchased from Biowhittaker (Walkersville, MD). Insulin (Bovine), and 17-beta Estradiol, Dimethyl Sulfoxide and Tamoxifen were purchased from Sigma Chemical Co (St Louis, MO). PPT, and DPN were obtained from Tocris (Ellisville, MO) I3C, DIM and Tryptophol were purchased from Aldrich (Milwaukee, WI). The sources of other reagents are either listed below or were of the highest purity available.

Cell Culture:
MCF 7 human breast adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). MCF 7 cells were grown in DMEM supplemented with 10% FBS (Biowhittaker), 10 ug/ml Insulin, 50 units/ml penicillin, 50 units/ml Streptomycin, and 2 mM L-Glutamine. The cells were grown to sub confluency in a humidified air chamber at 37 deg C containing 5% CO2. I3C, DIM, Tryptophol (99.9% HPLC grade) were dissolved in appropriate concentrations which were a 1000 fold higher than the desired concentration in the medium. 17 β Estradiol, PPT, DPN, Faslodex and Tamoxifen were also dissolved the same way at 1000 fold higher concentration than desired in sterile DMSO. DMSO was used as vehicle control for all experiments.

ERE assays:
Plasmid containing the consensus vitellogenin ERE in pgl2 vector was transfected according to manufacturer's instructions using Fugene 6 (Roche). 24 h later, the media was replaced with media containing 10% dextran charcoal stripped FBS. 24h later, the cells were treated with DMSO or 200 μM I3C for 48h. The cells were then treated with either DMSO, 10 nM 17 β Estradiol, 100 nM PPT, or 100 nM DPN. After 1 hour of treatment, cells were lysed and subjected to luciferase activity assays using the luciferase assay kit (Promega). The amount of protein was determined using the Lowry method, and the relative light units were normalized to protein input.

Western Blotting for ER α and ER β:
After the indicated treatments, cells were harvested in the media, pelleted by centrifugation at 8000 rpm for 5 min, resuspended in 1 ml PBS, and pelleted again by centrifugation. These cells were then resuspended in radio immunoprecipitation buffer (150 mM NaCl, 0.5% Deoxycholate, 0.1% NP40, 0.1% SDS, and 50mM Tris) containing protease inhibitors (50 ug/ml Phenylmethylsulfonyl fluoride, 10 ug/ml Aprotinin, 5 ug/ml Leupeptin, 0.1 ug/ml NaF, and 10 ug/ml b-Glycerophosphate). These extracts were then quantitated using the Lowry Method (Bio-Rad). Normalized amounts of protein were mixed with sample buffer [(25% Glycerol, .075% SDS, 1.25 ml b Mercaptoethanol, 10% bromophenol blue, 3.13% 0.5M SDS, and 0.4%SDS (pH 6.8)], and fractionated on 8% Polyacrylamide/0.1% SDS resolving gels using electrophoresis. Rainbow marker (Amersham Life Sciences, Arlington Hts, IL) was used as standards. Proteins were then transferred electrically to nitrocellulose membranes for an hour at 4degC. Equal loading was confirmed with Ponceau S incubation of membranes. The blots were then blocked for an hour in 5% Non fat dry milk dissolved in western wash buffer [10 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.05% Tween 20] at room temperature. The blots were the rinsed in plain western wash buffer and incubated overnight at 4 deg C in antibodies diluted in western wash buffer. Rabbit anti ER α and ER β antibodies (Santa Cruz biotechnology, Santa Cruz CA) were diluted 1:1000 in wash buffer. CDK2 immunoblotting was performed as described earlier (). Immunoreactive proteins were detected after incubation with horse-radish peroxidase conjugated secondary antibodies diluted to 3 x 10-4 in 1% NFDM in western wash buffer (Goat anti Rabbit IgG (Bio-Rad, Hercules, CA). Blots were then treated with enhanced chemiluminescence reagents (NEN Life Sciences), and all proteins were detected by exposure to Hyperfilm (Kodak).

RT-PCR

MCF 7 cells after the indicated treatment durations, were harvested in Trizol Reagent (Gibco?), and the recommended protocol was followed to extract total RNA. RNA was quantitated using spectroscopy and the quality of RNA was confirmed using A260/A280, and by electrophoresis on 1% Agarose gels. 2 ug of total RNA was subjected to Reverse Transcription using Mu-MLV Reverse Transcriptase (invitrogen) with random hexamers, dNTPs, and RNAse Inhibitor (Invitrogen). 4 ul of cDNA was then subjected to PCR using primers specific to ER α, ER β, PR, and GAPDH as described (15) The PCR products were run on 1% Agarose gels along with a 1 kb plus DNA ladder (Invitrogen).

Affinity Chromatography for ER-ERE binding:
The pbluescript plasmid containing the consensus vitellogenin ERE was subjected to restriction enzyme digest using Bgl II and Hind III. The products were electrophoreased on a 1% agarose gel containing ethidium bromide. The 550 bp band corresponding to the ERE was excised and extracted using the Qiaex II kit (Qiagen). About 7 ug of ERE fragment was biotinylated using Klenow enzyme as per manufacturer's protocol. ERE was extracted by ethanol/salt extraction. Overall, 16 ug of DNA was then used for the chromatography assay. MCF 7 cells treated with I3C or DMSO were lysed using a buffer

containing 10 mM Hepes, 150 mM NaCl, 1% NP 40, 10% glycerol, and 2mM EDTA, and a cocktail of protease inhibitors without DTT. Protein content of cell lysates were then normalized using the Lowry method (Bio-Rad, CA). Streptavidin conjugated agarose beads (Sigma) were resuspended in 400 μl of lysis buffer, and the column was divided into two eppendorf tubes with 200 μl each. About 2mg of total protein from DMSO or I3C treated cells was added to each column and incubated at 4 deg C for 1 hour with constant nutation. The column was then centrifuged at 4 deg C for 5 min at 5000 rpm. The unbound lysate was stored and the columns were washed using lysis buffer three times. The elution buffer was prepared by adding 400 mM NaCl to the lysis buffer and added to the beads. After incubation at room temperature for 5 min, the beads were centrifuged down and the supernatant was loaded and subjected to SDS-PAGE. The amount of bound estrogen receptors was assayed by immunoblotting as described above.

Results

a. Indole-3-Carbinol treatment selectively downregulates ER-α levels in MCF 7 cells.

It has been demonstrated that I3C treatment causes a marked arrest of MCF 7 cells in the G1 phase of the cell cycle. I3C has been shown to cause a decrease in the expression of cyclin dependent kinase 6 (CDK6) at the transcription level, as well as cause decreased kinase activity of CDK2. These effects are observed in ER- α positive and negative breast cancer cell lines. In addition, I3C and tamoxifen, cause an increased growth arrest of MCF 7 cells than wither drug alone, although by distinct mechanisms. Hence, to investigate if I3C had any effects on ER α and β levels in MCF 7 cells, these cells were treated with increasing doses of I3C for 48h and the expression levels of ER α and β was analyzed by immunoblotting. This duration was chosen because this has been shown to be optimal in causing G1 cell cycle arrest in MCF 7 cells. As shown in Fig 1(c), I3C treatment at increasing doses caused a selective decrease in levels of ER α protein, whereas ER β levels remain unaltered. It has been reported previously that 100 μM of I3C is the minimum dose effective in causing G1 cell cycle arrest of MCF 7 cells. This is also the minimum dose used that results in ER α downregulation. To study the kinetics of this downregulation, MCF 7 cells were treated with DMSO or I3C for varying durations and relative levels of ER α and β were studied by immunoblotting. Fig 1(a) shows a selective decrease in ER α levels with time and ER β remain unaltered. The kinetics of ER α downregulation is very similar to that of CDK6 downregulation, which was reported earlier (Fig 1(b)).

b. I3C downregulation of ER α occurs in the presence and absence of 17 b-estradiol and is not a consequence of G1 cell cycle arrest.

17 b estradiol has been shown to cause a downregulation of ER α by autoregulation. Hence to investigate if I3C induced downregulation of ER α is by such a pathway, MCF 7 cells were grown in media supplemented with 10% dextran charcoal stripped fetal bovine serum, and treated with I3C in the presence and absence of 17 b estradiol for 48 h. Relative levels of ER α and β were then analyzed by immunoblotting. As shown in fig 2,

I3C is more effective than 17 β estradiol in decreasing ER α levels. Also, I3C decreases ER α levels in the presence and absence of 17 β estradiol. It has been shown previously that ER α can physically associate with G1 cell cycle regulators, and can be phosphorylated by CDKs such as CDK2. Hence to address if ER α downregulation is a consequence of cell cycle arrest, MCF 7 cells were treated with 1 μM tamoxifen, a mixed antiestrogen shown to cause G1 cell cycle arrest in MCF 7 cells. Tamoxifen has also been known to cause increases in ER α expression. Results form fig 2 indicate that I3C downregulation of ER α is not a side effect of G1 cell cycle arrest in these cells, and that I3C can cause a decrease in ER α levels induced by tamoxifen.

c. I3C downregulation of ER α in MCF 7 cells is at the transcriptional level and I3C causes a decrease in ER α promoter activity:

I3C downregulation of CDK6 protein has been reported to be due to repression of transcription. Additionally, the kinetic profile between I3C downregulation of CDK6 and ER α is very similar. To test if ER α downregulation by I3C is also at the transcriptional level, MCF 7 cells were treated with DMSO, 200 mM I3C, 1 mM tamoxifen, a combination of tamoxifen and I3C, or 200 μM tryptophol for 48 h. Tryptophol is structurally very similar to I3C and has an extra carbon in its chain. This compound is ineffective in causing G1 cell cycle arrest and is used for indole structure specificity control. The cells were then subjected to RNA isolation and subsequent RT-PCR analysis using primers specific for ER α. The PCR products were electrophoreased on a 1% agarose gel and visualized using a UV transilluminator. Fig 1(c) shows an abrogation of ER α expression at the mRNA level by I3C in the presence and absence of tamoxifen. Tryptophol treatment has no effects on ER α mRNA levels indicating the specificity of I3C in ER α downregulation. Fig 3 shows that I3C treatment causes a marked decrease in the ER α promoter activity in MCF 7 cells transfected with the 3.2 kb region of the ER α in the pgl2 basic vector. This indicates that I3C causes an active repression of transcription of the ER α gene.

c. I3C does not alter the subcellular localization of ER β in MCF 7 cells.

As presented earlier in this section, I3C does not alter the total levels of ER b in MCF 7 cells. To test if the subcellular localization is affected by I3C treatment, MCF 7 cells were grown in media supplemented with 10% charcoal dextran treated FBS on cover slips. After 48 h of treatment with DMSO or 200 μM I3C, these cells were fixed using 10% PBS buffered formalin, and subjected to immunofluorescence using antibodies specific to ER β followed by incubation with FITC conjugated secondary antibodies. As shown in fig 3a, I3C treatment did not alter the predominantly nuclear localization of ER β in MCF 7 cells. This was confirmed by nuclear fractionation of DMSO and I3C treated MCF 7 cells and subsequent immunoblotting for ER β (data not shown). This is indicative that I3C might not alter ER β activity by causing changes in cellular localization.

d. I3C decreases functional ER α levels and increases functional ER β levels as assessed by binding to a consensus ERE in vitro:

As a step towards evaluating functional consequence of ER a downregulation induced by I3C, the relative binding of both estrogen receptors to their EREs was evaluated by an affinity chromatography column. Streptavidin-agarose beads were incubated with biotinylated ERE fragments obtained by restriction digest of vit-ERE-CAT constructs as described in materials and methods. Total cell lysates from DMSO or 200 μM I3C treated MCF 7 cells were incubated in these columns containing ERE conjugated beads. ERE bound proteins were eluted using a high salt solution, and this elute was electrophoreased on an SDS-PAGE gel, transferred to nitrocellulose membranes and blotted for ER α and β. Fig 5a shows that I3C downregulated functional ER α protein in MCF 7 cells, whereas I3C increased ER β to the ERE. This indicates a reversal in the functional ER α: ER β in MCF 7 cells by I3C treatment. This agrees well with the prevalent thought that increased levels of ER β: ER α in an estrogen responsive cell correlates with decreased cellular proliferation.

e. I3C causes a significant decrease in PPT, an ER α specific ligand induced ERE activity in MCF 7 cells.

A consensus vitellogenin ERE in pgl2 basic was introduced into MCF 7 cells grown in media supplemented with 10% charcoal dextran stripped FBS. The cells were treated with DMSO and with DMSO and 200 μM I3C for 24h. 24h later, the cells were treated with DMSO, 17 β estradiol, 200 μM I3C , E+I3C, 10 μM PPT, PPT+I3C, 10 μM DPN (ER β specific ligand) or DPN+I. 24h later, ERE activity was measured as relative light units and normalized to protein input. Fig 5(b) shows a robust increase in ERE activity with E2 treatment, and I3C effectively blocks this increase. PPT also shows a significant increase in ERE activity and I3C effectively blocks this as well. PPT has been shown in several systems to be extremely ER α specific possessing 400 fold higher affinity for ER α than ER β. DPN is an ER β specific ligand, and shows about 70 fold higher affinity for ER β than ER α. However, the optimal concentration of DPN where it only binds ER β needs to be determined in MCF 7 cells.

f. I3C treatment effectively blocks E2 induced progesterone receptor expression:

To test if I3C downregulation of ER α has functional consequences in MCF 7 cells, effects of I3C on E induced progesterone receptor expression were examined by RT-PCR. MCF 7 cells were grown in media supplemented with 10% charcoal dextran stripped FBS and were treated with DMSO and I3C in the presence and absence of 10 nM 17β E2 for 24 h. Total RNA was isolated from treated cells as described and subjected to RT-PCR with primers specific for progesterone receptor. The products were

visualized after electrophoresis on a 1% agarose gel containing ethidium bromide. Fig 6 shows the relative levels of PR mRNA. As expected, 10 nM E2 caused a robust increase in PR mRNA levels, and I3C treatment effectively blocks induction of PR transcription by E2. This indicates that I3C downregulation of ER α has functional consequences, possibly leading to downregulation of ER α induction of pro-proliferative genes.

Discussion

Epidemiological studies have shown that increased consumption of vegetables causes a decrease in cancer incidence in many different organs including the breast . Estrogen exposure has been shown to be strongly linked to breast cancer risk in women. Our experimental findings have shown that indole-3-carbinol (I3C) derived from cruciferous vegetables can inhibit growth of breast cancer cell lines in culture by decreasing expression and or activity of specific cyclin dependent kinases. I3C has also been previously shown to cooperate with the antiestrogen tamoxifen in causing growth of MCF 7 cells in culture. We describe a novel effect of I3C on the expression and activity of the estrogen receptors α and β. I3C treatment, at doses effective at growth arrest, shows a selective downregulation of ER α in MCF 7 cells. I3C causes a dose and time dependent decrease in ER α expression. This downregulation is at the transcriptional level, and I3C causes a decrease in ER a promoter activity. Our results also indicate that this effect is specific to I3C, is not blocked by tamoxifen, and that I3C does not block E2 downregulation of ER α. We also demonstrate that the downregulation of ER α by I3C leads to lowered binding of ER α to the ERE as well as to reduction of E2 induced ERE activity. Treatment with I3C also leads to blocks E2 mediated induction of estrogen target genes such as progesterone receptor (PR).

A high level of functional ER α relative to functional ER β has been shown to correlate with high levels of proliferation in multiple estrogen sensitive tissues. During lactation, a phase with minimum proliferation of mammary epithelial cells, ER β levels are elevated. ER β null mice show marked increase in proliferation of lactating mammary epithelial cells, indicating a role for ER β in inhibition of proliferation. Hence increased ER β levels are required for mammary terminal differentiation. Overexpression of ER β in MCF 7 and T47D have shown a marked decrease in growth rates of these cells. Our results demonstrate a reversal of levels of functional ER α to ER β, correlating well with its growth arresting effects.

The exact mechanism of I3C downregulation of ER α is presently unknown. However, our results show that mechanism of ER α downregulation by I3C may differ from E2 induced ER a downregulation. E2 treatment causes a downregulation of both ER α and ER β in most E2 responsive cells, and I3C does not inhibit E2 downregulation of ER α. In addition, tamoxifen is unable to block the I3C induced downregulation of ER α. Most importantly, E2 downregulation of ER α in MCF 7 coincides with a large mitogenic effect of E2 on these cells, whereas, I3C causes a marked growth arrest. Taken together, I3C downregulation of ER α may be distinct from other known mechanisms such as autoregulation of ER α by E2.

Previous studies with I3C have shown that cyclin dependent kinase 6 (CDK6) is transcriptionally downregulated in I3C treated MCF 7 and MDA-MB-231 breast cancer

cells. I3C treatment has been shown to disrupt Sp1/Ets transcription factor binding to their consensus binding site on the CDK6 gene promoter. Interestingly, the kinetics of this downregulation coincides with the kinetics of ER α downregulation by I3C. Moreover, the minimum region in the ER α promoter needed for gene expression has been determined, and shown that a Sp1 site in this region is needed to transcribe ER α gene effectively. It is possible that I3C treatment leads to disruption of Sp1 binding at this site as well, leading to ablation of ER α expression.

Functionally, our results indicate that I3C selectively ablates function of ER α by disrupting the binding of ER α to the ERE. Use of ER α specific ligand such as PPT led to an ERE activity comparable to that of E2. I3C when coadministered with PPT showed a marked decrease in ERE activity, and this combination led to ERE activity comparable to I3C treatment alone. Use of ER β agonist DPN also showed an increase in ERE activity as expected, and I3C inhibited this increase to a lesser extent compared to I3C +PPT group. **The optimal dose of DPN needs to be determined.** Consistent with the I3C effects on ERE activity observed, our results also show that I3C blocks the 17 β estradiol induction of estrogen responsive genes such as progesterone receptor (PR) at the transcriptional level.

Previous work has established the ability of I3C to block cell growth in estrogen responsive MCF 7 and estrogen unresponsive MDA-MB-231 breast cancer cells. The results presented here demonstrate that I3C can also cause selective downregulation of ER α expression and activity in MCF 7 cells. Given the vast heterogeneity of epithelial cell phenotypes in human breast cancer in vivo, I3C may be a promising agent that is an effective cytostatic drug, and decreases activity of ER α in ER α positive sub population, thus potentially retarding the malignant progression of these cells.

Reportable Outcomes:

Not Applicable

Conclusions:

Please refer to the discussion section.

Appendix:

Fig 1(a) : Time-Course experiment testing effects of I3C on ER-α and ER- β

Western blot of extracts from MCF-7 cells treated with I3C over increasing times. Cells were treated with either vehicle control DMSO (D) or 200 mM I3C (I) for 0, 12, 24, 48 and 72 hrs. SDS-PAGE and electro-transfer was carried out as described in Fig1.

Fig 1(b) :Dose-dependent effects of I3C on the estrogen receptors.

Western blot of extracts from MCF-7 cells treated with increasing concentrations of I3C. Extracts were run on a 10% SDS-polyacrylamide gel and electrophoresed till the bromophenol blue dye reached the bottom of the gel. The proteins were then electrically transferred to a nitrocellulose membrane for 1 hour after which the membrane was blocked in 5% milk for 1 hour. The membranes were then probed with anti ER-α or anti ER-β primary antibodies overnight followed by 3 washes of 20 min each (in 1X Tris buffered saline containing Tween-20) and then incubated with anti-rabbit secondary antibodies conjugated to horseradish peroxidase enzyme. Protein bands were visualized using an enhanced chemiluminiscence (ECL) kit and X-ray film. CDK2 is used as a loading control as its total protein expression has been shown to be unaltered by I3C treatment in MCF 7 cells.

Fig 2: Down-regulation of ER-α by I3C alone and in combination with tamoxifen is independent of estradiol:

Western blot of MCF-7 cells treated with DMSO (D), 200 mM I3C (I), 1 mM tamoxifen (T) and a combination of tamoxifen and I3C (T+I) for 48 hrs either in the absence (-) or presence (+) of 10 nM estradiol (E2). Cells were starved for 24 hours in Dulbecco's Modified Eagle Medium (DMEM) without phenol red and to which was added 10% charcoal dextran stripped fetal bovine serum. After 24 hrs, treatment with the respective drugs was initiated for 48 hrs in the same stripped medium as above but to which either 10 nM estradiol or an equivalent amount of vehicle (DMSO) was added. Cells were harvested and extracts prepared for western blotting

Fig 3 (a): RT-PCR (ER-α, ER β, GAPDH)

MCF-7 cells were treated with either DMSO (D), 200 mM I3C (I), 1 mM tamoxifen (T), tamoxifen and I3C (T+I) or 200 mM tryptophol (tryp) for 48 hours. RNA was extracted using the Trizol method after which 2 mg RNA was reverse-transcribed to make cDNA. 400 ng cDNA was amplified by PCR using either ER-α or GAPDH specific primers as described in the results section. The total PCR reaction (50 ml) was then loaded onto a 1% agarose gel containing ethidium bromide and then visualized on a UV trans-illuminator.

Fig 3(b). I3C causes a decrease in ER-α promoter activity.

MCF 7 cells were transfected with a construct containing the 3.2 kb region of the ER-α promoter region fused to the luciferase gene in a pgl2 basic vector. The cells were then treated with DMSO or 200 mM I3C (I) for 24h followed by lysis, and measurement of luciferase activity as per manufacturer's protocol. Normalized relative luciferase activity is presented in this figure.

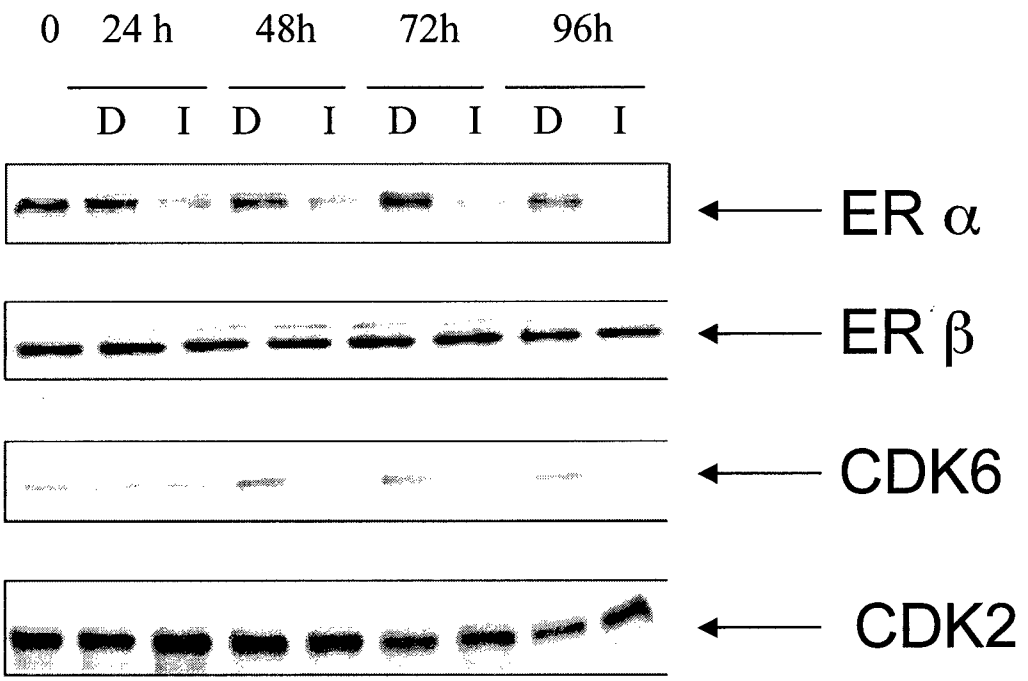
Fig 4. I3C does not change the subcellular localization of ER-b in MCF 7 cells.
MCF 7 cells were grown in 8 well slides and treated with DMSO (D) or 200 mM I3C (I) for 48h. The effects of I3C on the nuclear localization of ER-β was evaluated by indirect immunofluorescence as described in materials and methods.

Fig 5 (a). I3C treatment causes decreased ER a binding to Vitellogenin ERE, and increased binding of ER b to the ERE.
MCF 7 cells were treated with DMSO or 200 mM I3C for 48 h. Cell lysates were passed through agarose beads conjugated with biotinylated EREs obtained by restriction digest from vit-ERE-CAT as described in materials and methods. Elution with a high salt solution was followed by immunoblotting for ER a and b.

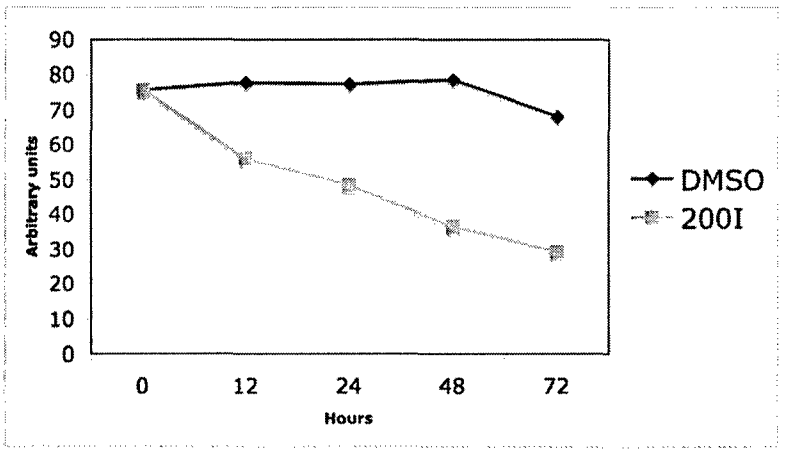
Fig 5(b). I3C treatment causes a decrease in ERE activity
MCF 7 cells were transfected with the pgl2 vector or vector containing the consensus ERE of the vitellogenin gene. 24h later, the cells were treated with the DMSO or 200 mM I3C in media containing charcoal dextran stripped fetal bovine serum. 24 h later the cells received indicated treatments: DMSO, 10 nM E2, 200 mM I3C, E2+I, 10 mM PPT, PPT+I, 10 mM DPN or DPN+I. 24 hours later, the cells were lysed and relative luciferase activity was determined as described in figure 8.

Fig 6. I3C inhibits E2 induction of the progesterone receptor mRNA in MCF 7 cells.
MCF 7 cells grown in media containing charcoal dextran stripped serum were treated with DMSO (D), 200 mM I3C (I), 10 nM b-estradiol (E2), or a combination of E2 and I. 24h later, RNA was isolated as described in the materials and methods section, and subjected to RT-PCR using primers specific to progesterone receptor mRNA. The RT-PCR products were electrophoreased on 1% agarose gels containing ethidium bromide and visualized using a UV trans-illuminator.

Fig 1a



ER α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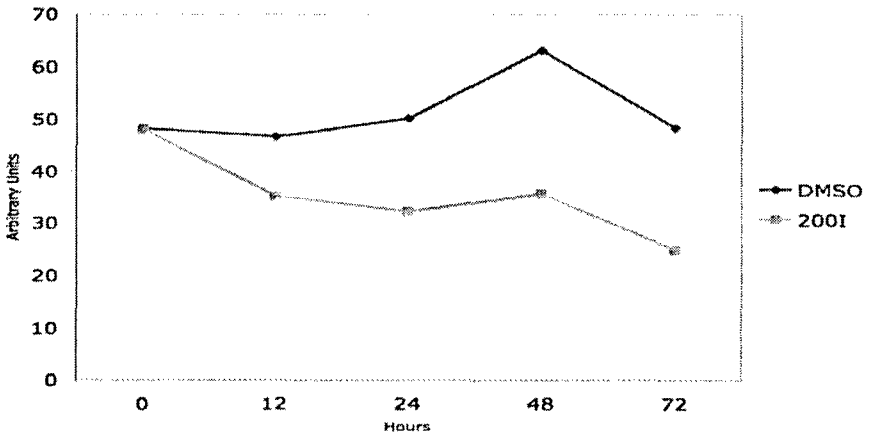
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200I

ER β

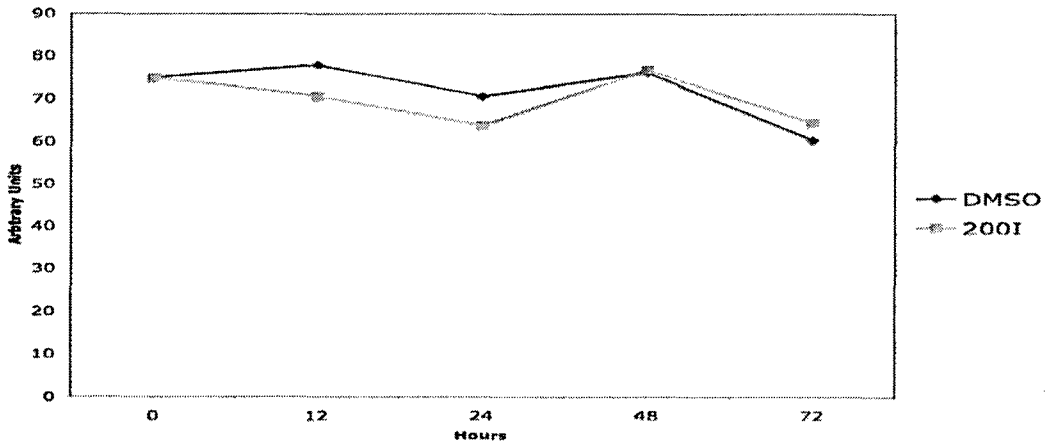

90
80
70
60
50
40
30
20
10
0
Arbitrary Units
0
12
24
48
72
Hours
DMSO
200I

Fig 1 b

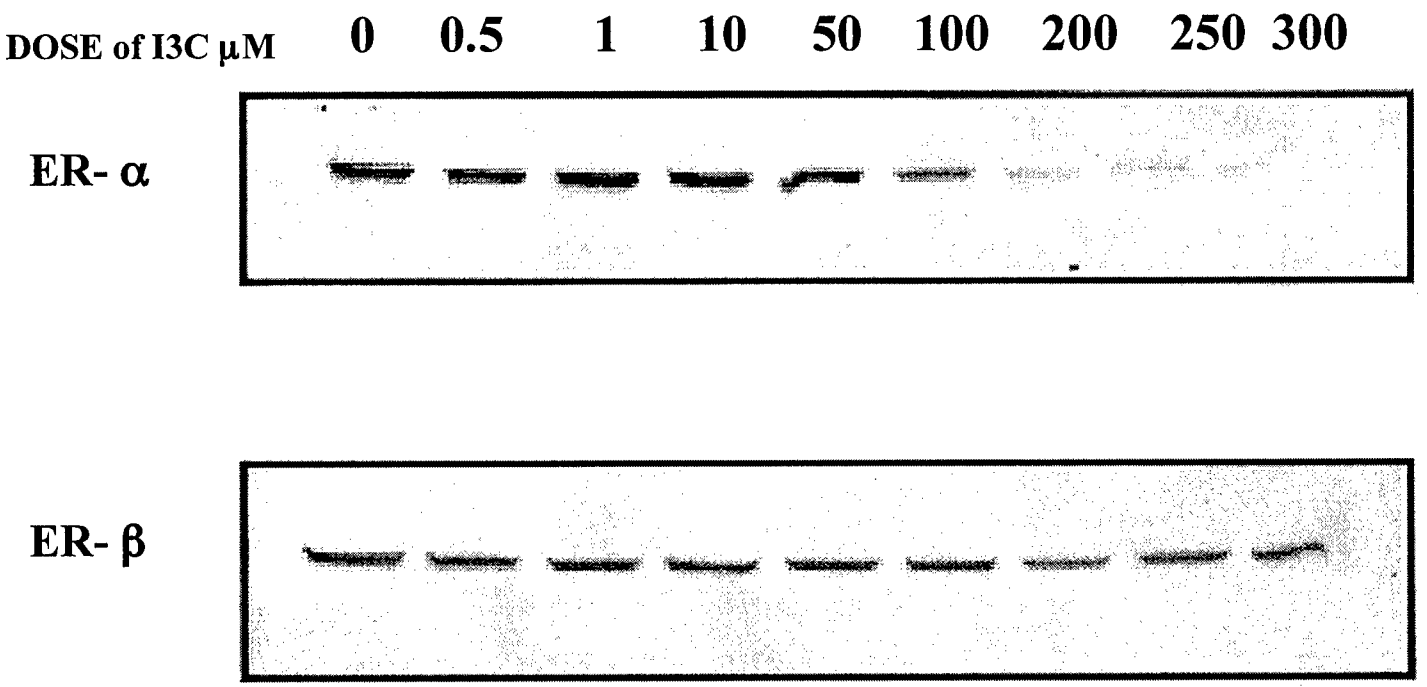


Fig 2

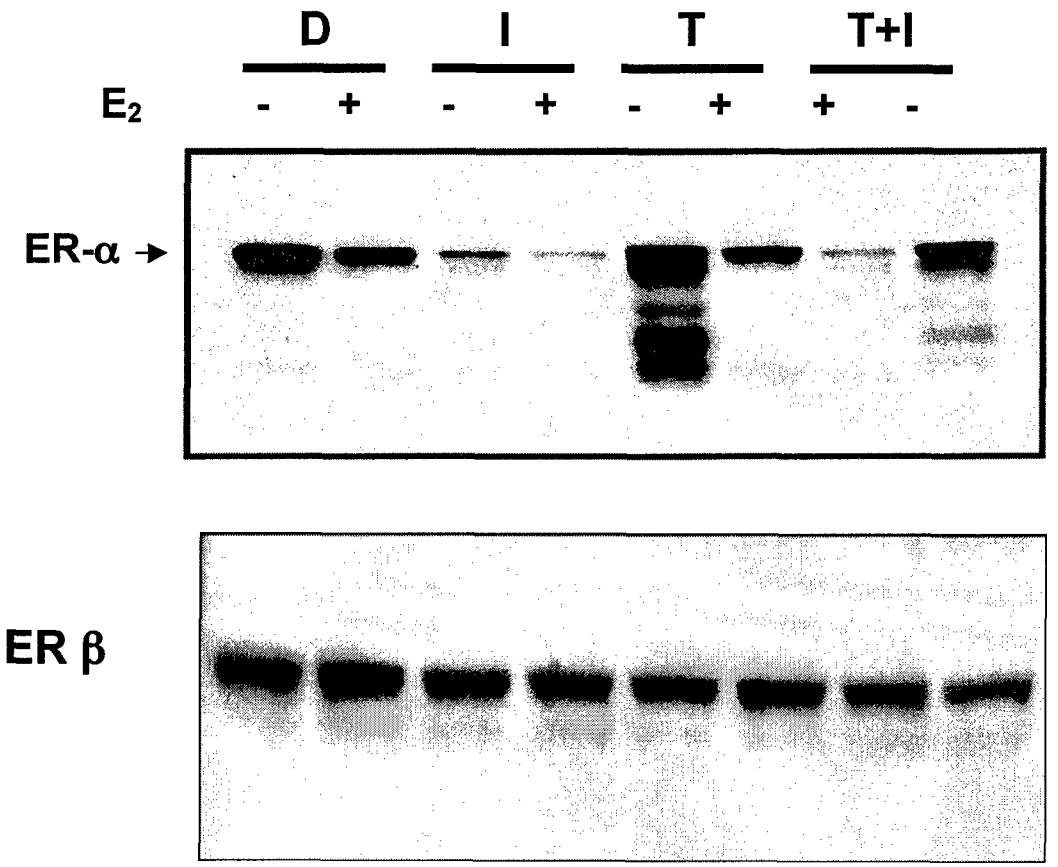


Fig 3(a)

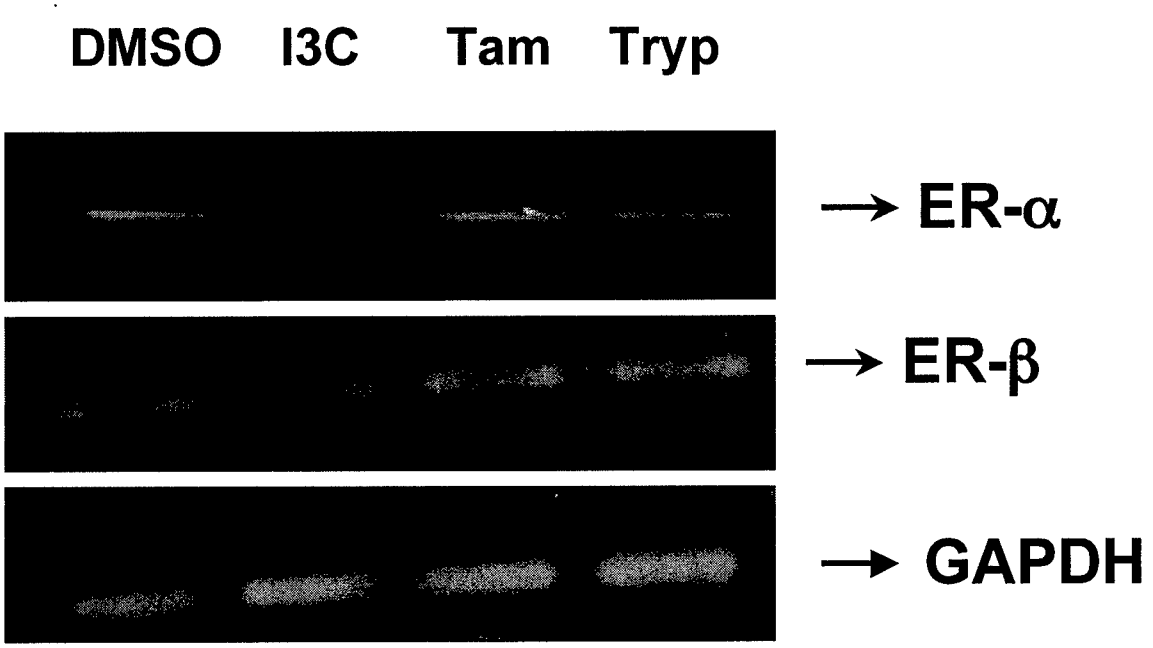


Fig 3(b)

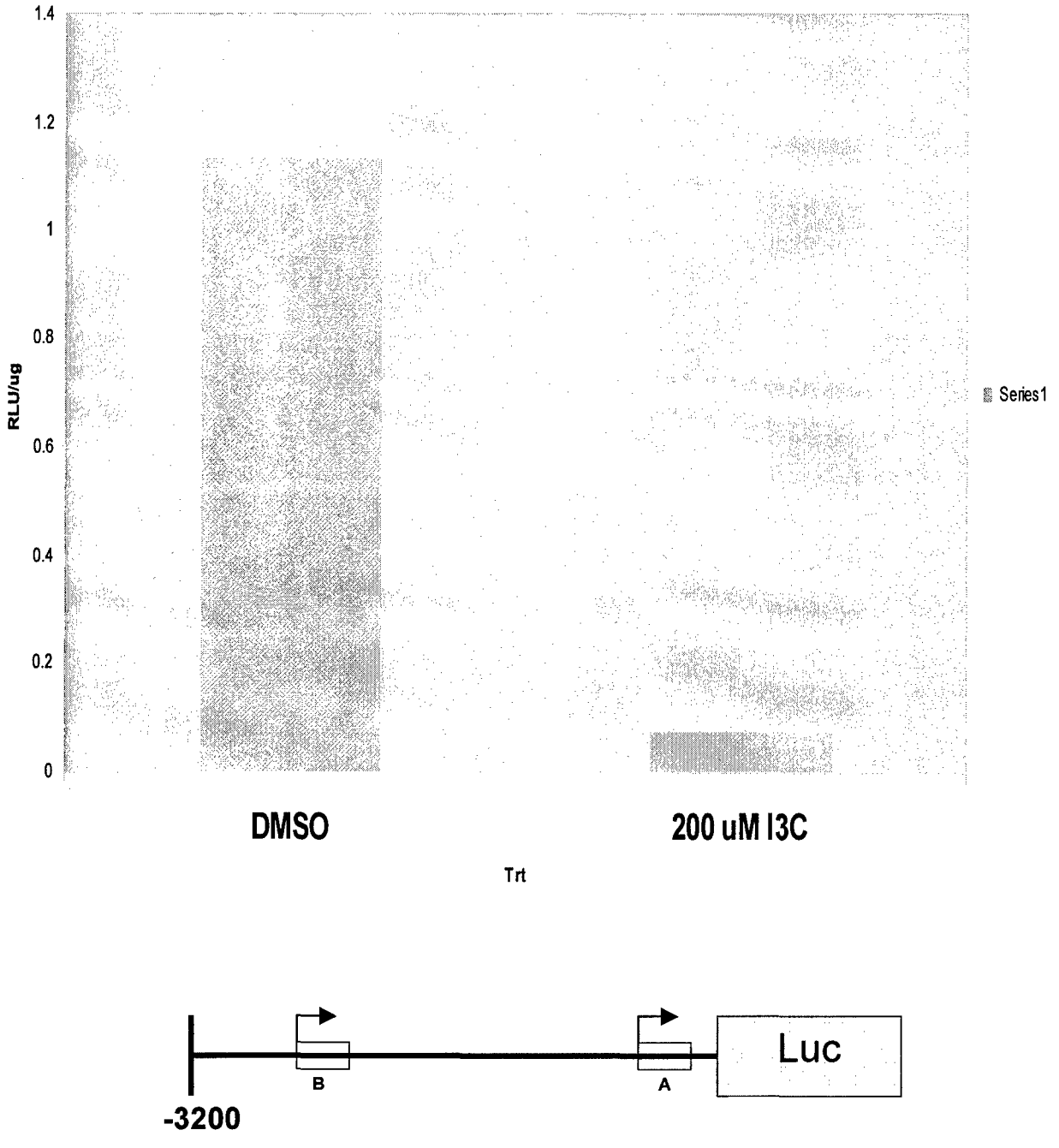
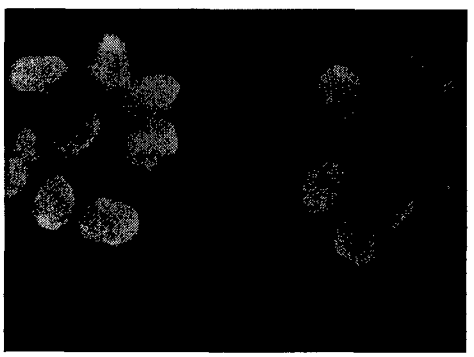
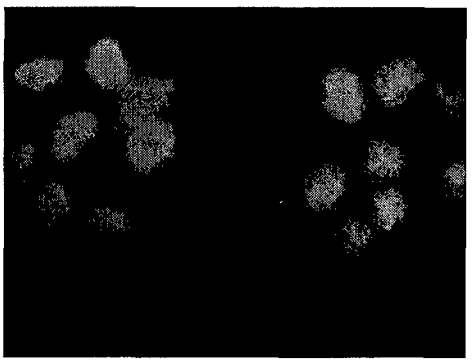
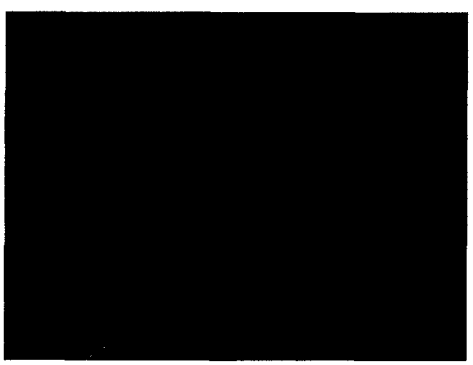

1.4
1.2
1
0.8
0.6
0.4
0.2
0
RLU/ug
Series1
DMSO
200 uM I3C
Trt
B
A
Luc
-3200

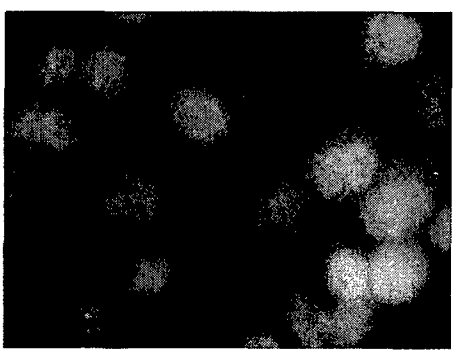
Fig 4





DMSO





I3C

Fig 5(a)

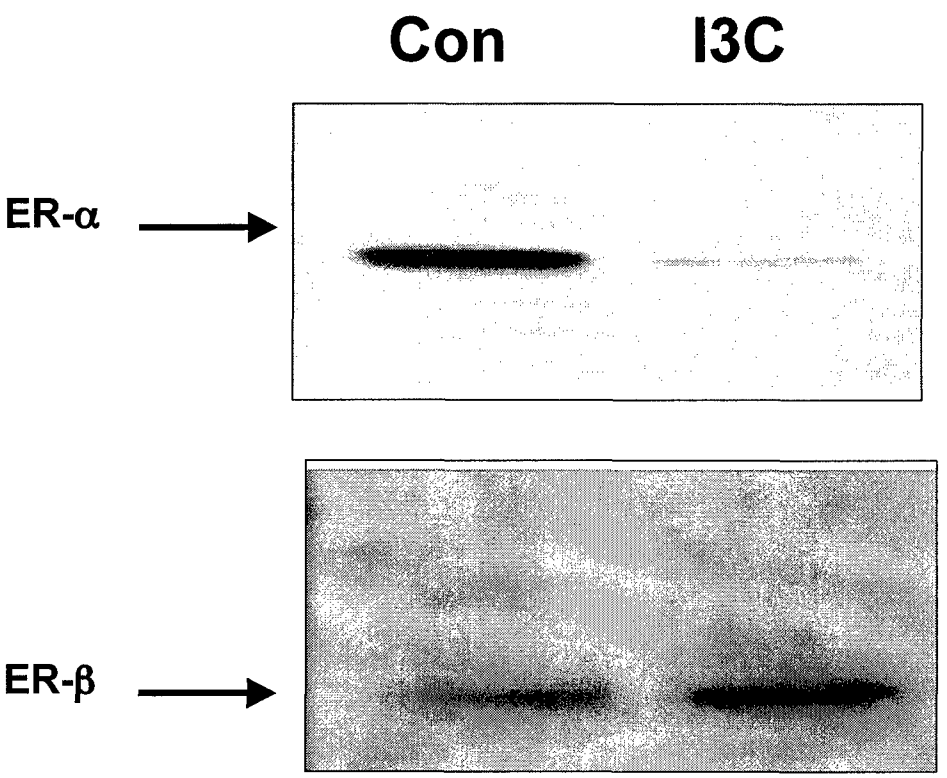


Fig 5 (b)

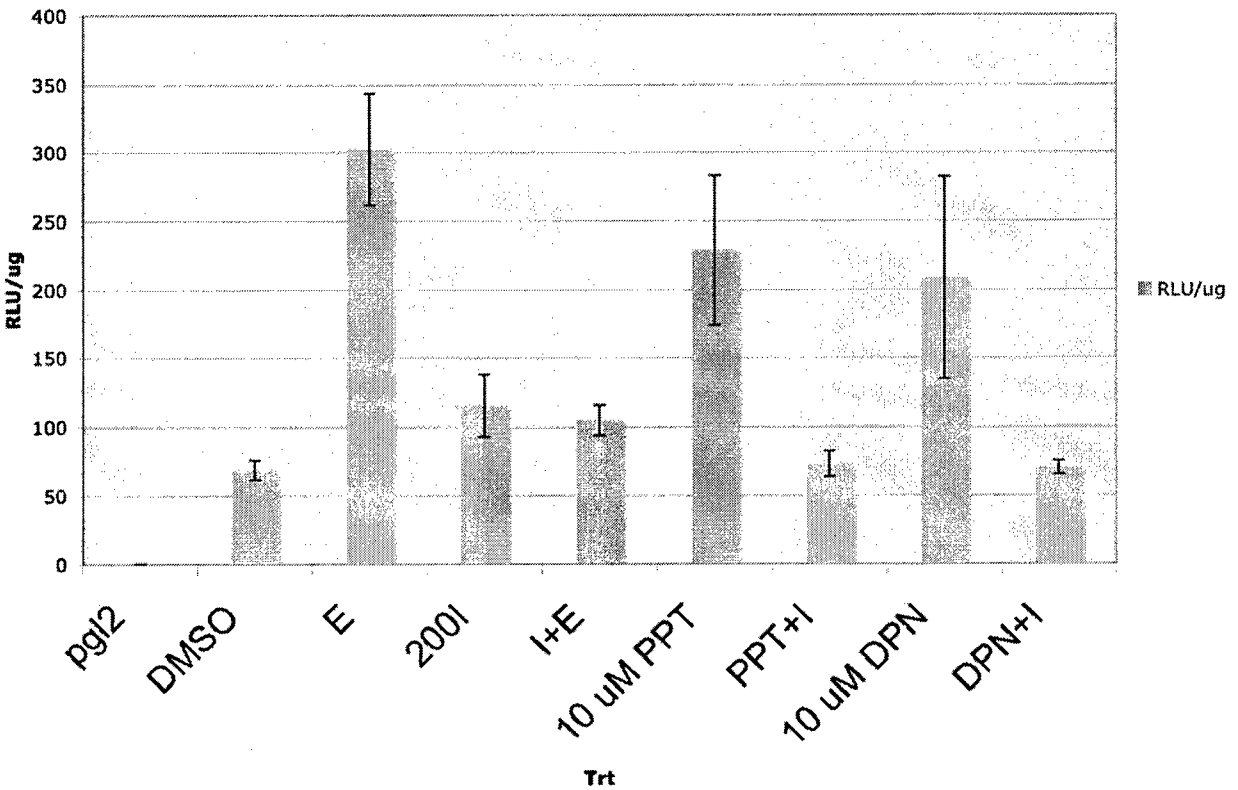

400
350
300
250
200
150
100
50
0
RLU/ug
pgl2
DMSO
E
200I
I+E
10 uM PPT
PPT+I
10 uM DPN
DPN+I
Trt
RLU/ug

Fig 6